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Sex Determination in Southern Flounder, *Paralichthys lethostigma* From the Texas Gulf Coast and Implications of Climate Change

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Sex Determination in Southern Flounder, *Paralichthys lethostigma* From the Texas Gulf Coast and Implications of Climate Change

by

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Dedication

This work is dedicated to my mother and father who taught me to always work my hardest and give my best, regardless of the outcome, and supported my love for nature, no matter what type of trouble it got me into or what kind of strange critters I brought home.

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Abstract

Sex Determination in Southern Flounder, *Paralichthys lethostigma* From the Texas Gulf Coast and Implications of Climate Change

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In marine flatfish of the genus *Paralichthys*, temperature plays a large role in sex determination. Thus, global climate change could have significant effects on southern flounder (*Paralichthys lethostigma*), a commercially and recreationally important flatfish whose populations have steadily declined in Texas in the last 25 years. The most susceptible areas to global climate change are shallow water environments, particularly estuaries, which serve as essential nursery habitats for juvenile southern flounder. While in the estuaries, juveniles develop, and sex is determined. Juvenile southern flounder possess genotypic sex determination; however, the sex of females is highly influenced by temperature and can result in sex reversal. The temperature-sensitive enzyme complex

responsible for estrogen biosynthesis in vertebrates is aromatase cytochrome P450 ($P450_{arom}$), a critical component in ovarian differentiation that can be used to measure presumptive males and females exposed to a gradient of temperatures. This research identifies that sex is influenced by temperature between 35 and 65 mm total length (TL) and establishes that increases in temperature from 18 °C during this size range produce increasingly male skewed sex ratios in southern flounder from Texas. The findings presented here are critical for optimizing production of females in culture and for developing stock enhancement programs of southern flounder in Texas.

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INTRODUCTION

Southern flounder (*Paralichthys lethostigma*) is a commercially and recreationally important flatfish whose wild populations have steadily declined since the mid 1980's throughout the Midwest Atlantic and Gulf of Mexico (Daniels, 2000; GSMFC, 2000). These declines are primarily attributed to increased fishing pressure and habitat loss (Takade-Heumacher and Batsavage, 2009; Wenner and Archambault, 2005). Southern flounder spawn offshore at depths of 20-60 m during winter months (Stokes, 1977). Currents transport the larvae to shallow water estuaries where they settle out at approximately 8-12 mm (TL) and begin life on the bottom (Nañez-James et al., 2009; Glass et al., 2008; Wenner and Archambault, 2005). In Texas, recent management restrictions have included a reduction in bag limit from 5 to 2 fish and prohibition of "gigging" during the month of November. It has been proposed that flounder emigration through the passes to offshore for spawning is highest in November (Stokes, 1977). Due to elevated demand for southern flounder and the apparent decline in wild stocks, both the necessity and initiatives to implement stock enhancement programs have increased (Faulk and Holt, 2009).

While the natural range of southern flounder is from North Carolina to the western Gulf of Mexico, they are discontinuous around the southern tip of Florida

(Daniels, 2000; Blandon et al., 2001; Wenner and Archambault, 2005). This separation in wild stocks has led some researchers to propose that there are population differences between Atlantic and Gulf stocks of southern flounder (Daniels, 2000). In addition, genetic analysis has determined that there are significant differences in allele frequencies in southern flounder stocks between major bay systems along the Texas coast (Blandon et al. 2001), and populations may possess the ability to adapt to localized environmental conditions.

Flatfish of the genus *Paralichthys* share a commonality in the means by which they differentiate into males and females. Paralichthid flounder exhibit a temperature sensitive form of genetic sex determination (GSD) commonly misinterpreted as temperature dependent sex determination (TSD), a form of environmental sex determination (ESD), where phenotypic sex is determined by water temperature (Luckenbach et al., 2003). Though TSD has been reported in over 50 fish species, most of these cases have been reported from laboratory studies, and are likely not true TSD, but rather GSD with an influence of temperature (Conover and Kynard, 1981; Conover, 2004).

In the case of southern flounder, laboratory studies have shown that females and males are genetically XX and XY respectively, and that sex determination in males is purely genetic, but sex can be reversed by temperature in females (Luckenbach et al., 2004). It may be difficult to identify the true mechanisms determining sex among species due to difficulties in mimicking the natural environmental variability in the laboratory. Thus, if the mechanisms determining sex are not well understood, it may be harder to

predict naturally occurring sex ratios. Currently, GSD with a temperature influence on sex determination in the wild has been shown to persist only in fishes from the family Atherinidae (*Menidia* sp.) (Conover and Kynard, 1981), where there are latitudinal differences in the degree of temperature influence on sex. It has been proposed that GSD plays a larger role in sex determination of fishes among populations from northerly latitudes, while more southerly latitudes exhibit GSD with a higher degree of temperature influence (Lagomarsino and Conover, 1993). There may be an adaptive significance of these differences towards optimizing reproductive success as suggested by Conover, (1984), but research is currently limited in this particular area.

Recent studies have shown a distinct pattern of GSD with an influence of temperature in southern flounder from North Carolina where both high (28° C) and low (18° C) water temperatures produce male skewed sex ratios, while an intermediate temperature (23° C) produces an approximate 1:1 sex ratio of males and females (Luckenbach et. al., 2003). The critical period where sex is influenced by temperature has not yet clearly been defined in southern flounder. It has been proposed that this critical period occurs between ~40 mm and 120 mm total length (TL), and histological analysis has shown that tissue differentiation begins at ~75 mm total length at approximately 4 to 6 months of age in southern flounder populations from North Carolina (Luckenbach et al., 2003). The enzyme complex responsible for estrogen biosynthesis in vertebrates is cytochrome P450 aromatase ($P450_{arom}$), which is a critical component in ovarian differentiation (Crews, 2003; Luckenbach et al., 2005). Kitano et al. (1999) found that for proper testicular differentiation to occur in Japanese flounder (*Paralichthys*

olivaceus), $P450_{arom}$ mRNA must be maintained at extremely low levels while increased levels must be maintained for ovarian differentiation.

The expression of $P450_{arom}$ was found to be an accurate measure of ovarian differentiation in southern flounder before sex can be histologically verified (Luckenbach et al., 2005). Juveniles differentiating into females exhibited high levels of $P450_{arom}$, while juveniles differentiating into males exhibited low levels of $P450_{arom}$. Since $P450_{arom}$ is a temperature sensitive enzyme complex, it can be used to measure presumptive males and females exposed to a gradient of temperatures (Frederieke et al., 2005; Luckenbach et al., 2005).

Global temperatures are changing at a more rapid rate than shown by historical records, and the potential of widespread negative impacts has increased (IPCC, 2007). While many of the causes of global climate change are poorly understood, recent models show a significant increase in global temperature in the last decade. Furthermore, for North America, models have projected air temperatures to increase ~2°C to 4°C throughout the next century (Intergovernmental Panel on Climate Change [IPCC] Synthesis Report, 2007). While this rise in temperature may seem minor, it may significantly affect many ecosystems. Changes in global climates can affect water temperature, currents, sea levels, the amount of water vapor that enters the atmosphere, precipitation and much more (IPCC, 2007). In addition, greenhouse gasses coupled with chlorofluorocarbons (CFC's) can increase the rate at which the global climate can change (Sala et. al., 2000). These changes in climate can indirectly alter ecological interactions between predator and prey by increasing mortality, changing behaviors, rates of

development, distribution, or trophic shifts (Holt, 2002). Such changes in climate can also lead to habitat loss (Walther et al., 2002). This presents a significant problem when certain species may be dependent on a particular habitat for survival. Two of the most vulnerable areas susceptible to global climate change are shallow water environments, particularly estuaries, and coral reefs (IPCC, 2007). According to recent literature, temperatures have already risen ~0.8° C globally (IPCC, 2007), and yet there is limited research looking at the effects of global climate change on the early life stages of fish. Consistently warmer winters along with other effects of global climate change may have major effects on larval fish populations in shallow water coastal habitats. (Kleypas and Hoegh-Guldberg, 2008; O'connor et al., 2007). Adults of most species are typically more tolerant of these changes, while early developmental stages are often more sensitive leading to developmental abnormalities or even increased rates of mortality.

This study investigates the size range at which temperature plays a role in the sex determination of juvenile southern flounder, as well as how sex ratios vary across water temperatures characteristic of the Texas coast during the spring, when juvenile flounder are in shallow coastal estuaries. I hypothesize that due to latitudinal differences in temperature regimes of Atlantic flounder stocks, I will see a difference in the resulting juvenile sex ratios when exposed to temperatures representative of Texas estuaries. Additionally, this study aims to further define the critical period which temperature influences sex determination in southern flounder.

MATERIALS AND METHODS

Experimental Design

In the fall of 2009, adult southern flounder were collected from areas in Aransas Bay adjacent to Port Aransas, Texas. The adult flounder were relocated to the Fisheries and Mariculture Laboratory of the University of Texas at Austin Marine Science Institute where they were maintained in 36 kL recirculating raceway systems (Faulk et al., 2007). Adult southern flounder spawned naturally under a manipulated temperature and photoperiod (Arnold et. al., 1977). Salinity and temperature were maintained at 19 ± 1°C, 33 ± 1 ppt and a D.O. ≥ 6.0 mg/L respectively. A total of four spawns were collected from late November through December to obtain enough fish for the study. Eggs were incubated with gentle aeration in 265 L recirculating larval rearing tanks which were held at 18 °C for the duration of larval rearing. After hatching, larvae were reared as described in Faulk and Holt (2009) through post metamorphosis.

At 35 dph, fish were fed a 250 μm pelleted micro diet (Otohime, Reed Mariculture, Camplbell, CA, USA), and pellet size was increased as the fish grew up to 1410 μm. Juveniles requiring larger pellets were fed a 2.0 mm micro diet (PI Granules, Zeigler, Gardners, PA, USA) through the end of the study. The amount of food fed per tank was determined from a previous study by Faulk and Holt, 2009. All pelleted micro diets were dispensed continuously during daylight hours by use of Automatic Microdiet Dispenser (AMD) System (Department of Fisheries, Government of Western Australia, AUS), or lifeguard automatic feeders (Lifeguard Aquatics, Cerritos, CA, USA).

Temperature, salinity, and dissolved oxygen were checked and maintained throughout the duration of the study, and residual feed and waste was siphoned from each tank each day.

Juveniles were sorted into 2 separate initial size groups of 35-40 mm (group 1) and 65-70 mm (group 2) (TL (I)). For group 1, 35 randomly selected individuals were placed into 10 separate 265 L round plastic tanks partially submerged in 5 independent 4500 L reservoirs (2 -265 L tanks/reservoir). The same design was used for group 2 except that 25 individuals were placed into each 265 L tank. There were 2 replicate 265 L tanks for each temperature treatment (18, 21, 24, 27, 30 °C) and size group totaling 20 tanks. Water from the 4500 L reservoir circulated through the submerged tanks, a biofilter, sand filter, and heat pump to maintain temperature (± 0.5 °C) and water quality. HOBO Waterproof Temperature/Alarm Pendant Data Loggers (Onset, Pocasset, MA, USA) were deployed a week prior to beginning the experiment and logged temperature for the duration of the experiment. Initially, each 4500 L reservoir was maintained at 18 °C and fish were added to the tanks. After a 24 h acclimation period, fish were either maintained at 18 °C or gradually raised to 21, 24, 27, or 30 °C at a rate of 3 °C per day.

Rulers of known length (159 mm) were added to the tanks, and photos were taken of fish in each tank adjacent to the rulers to measure TL every other week using ImageJ v 1.34u software (http://rsb.info.nih.gov/ij/). This technique scaled the object of known length and measured the approximate length of each fish on a computer allowing measurements without disturbing the fish during the experiment. Juvenile southern flounder were reared until all fish in each tank were at least 80 mm TL across all

temperatures (approximately 180 days) in order to histologically identify the gonads from each fish sampled.

At this time, 20 individuals were randomly sampled from each replicate tank. Following sampling, fish were anesthetized in a solution of iced seawater and clove oil at a concentration of 20 ppm. After a minute in the solution, the fish were removed, photographed, measured, and a scalpel was used to quickly sever the spinal cord. Due the small size of the gonads, each fish was carefully dissected under a microscope, and the gonads were removed. All dissecting tools were sterilized and rinsed between extractions. One gonad was taken for histology and the other for quantitative real time polymerase chain reaction (qRT-PCR).

qRT-PCR

Gonadal samples were placed in RNA*later* (Applied Biosystems/ Ambion, Austin,TX, USA) and kept at -20 °C until processing. Immediately prior to processing, gonadal tissue was removed with finely tipped forceps and gently blotted to remove excess RNA*later* solution. Samples were then placed in 2.0 mL tubes with 1.0 mL of TriReagent© (Applied Biosystems/ Ambion, Austin, TX, USA) and 0.5 ml of zirconia homoginization beads. Samples were homogenized in a vortex genie bead mill for a minimum of 10 min, and those that did not completely homogenize were run for 5 additional min until completely homogenized. In some cases, connective tissue within the sample did not completely homogenize after additional time in the bead mill. Total mRNA was extracted from each sample following the TriReagent© manufacture's

protocol. RNA concentration was measured in duplicate using a NanoDrop© ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington DE, USA). Purity of samples (A260/280) ranged from 1.76-2.01.

$P450_{arom}$ mRNA was quantified using a two-step quantitative real-time PCR (qRT-PCR). For this method, 500 ng RNA was reverse transcribed to cDNA using the Verso™ cDNA synthesis kit (Thermo Fisher Scientific, USA) following manufacture's protocol. The qRT-PCR was run on a Stratagene MX3000P (Agilent Technologies, Santa Clara, CA, USA). The Maxima© SYBR green qPCR kit (Fermentas, Glen Burnie, MD, USA) was used with gene specific primers for $P450_{arom}$ from Atlantic croaker (*Micropogonias undulatus)*, which were subsequently modified based on the published sequence of $P450_{arom}$ in southern flounder (Accession: AY902192, GenBank, http://www.ncbi.nlm.nih.gov/genbank/). The primers were (sense: ACCTGGCGATGACTCCCGTA and antisense: TGCCCTTTGGTACCCTGTAG). The total reaction volume was 25 μl containing 1X Maxima© SYBR green qPCR master mix with ROX, 200 nM each primer and 2 μl of cDNA template. The thermal program began with initial enzyme activation at 95 °C for 10 min, and proceeded with denaturing for 30 s at 95 °C, annealing for 28 s at 58 °C, extension for 30 s at 72 °C, and ran for 40 cycles. A single-cycle melting curve from 55 to 95°C was performed following each qRT-PCR reaction which exhibited only a single peak in all runs. The qRT-PCR product was cloned and sequenced as described in Faulk et al. (2010) and confirmed as southern flounder P450 aromatase using BLAST search (www.ncbi.nlm.nih.gov/blast).

A seven point plasmid standard curve containing the P450arom insert was created with the highest copy density at $2.34e^{7}$ diluted 10-fold to $2.34e^{1}$ in order to quantify the number of $P450_{arom}$ copies in each sample. Amplification efficiency for the plasmid standard ranged between 93.4 and 101.8%. A no template control (NTC) was also included in each 96 well plate and all reactions were performed in duplicate. The primers were also tested with RT- controls without any detectable signal.

Ct values from qRT-PCR were plotted into the standard curve to calculate relative amounts of $P450_{arom}$ per sample. The remaining cDNA from each sample was used for absolute quantification using a Quant-iT PicoGreen dsDNA quantification kit (Invitrogen Molecular Probes, Cambridge, UK) according to manufacturer's protocol. Blanks, standard curves, and samples per plate were run in duplicate. SPECTRAmax GEMINI XS microplate flourometer and SOFTmax PRO Software (Molecular Devices, USA) were used to create a standard curve, and the average absorbance values for each sample were plotted against the DNA standard curve. The relative amount of $P450_{arom}$ obtained for each sample from qRT-PCR was divided by the cDNA concentration obtained from the Quant-iT kit to normalize $P450_{arom}$ mRNA expression among samples, and are reported as copies of $P450_{arom}$ per μ cDNA (Libus and Štorchová, 2006)

Histology

To verify the results of the normalized qRT-PCR, histology was performed on 50 randomly selected fish. Gonadal tissue samples were placed in a 1.5 mL vial containing a 10% formalin solution immediately following dissection until histological processing.

Randomly selected tissues were then washed with deionized water and stored in 2mL of a 30% sucrose in PBS solution at 4 °C for at least 12 hours or until the tissue sank to the bottom of the vial. Samples were placed in a plastic mold and vertically embedded in OCT gel medium on dry ice until thoroughly frozen. Samples were stored at -80 °C thereafter. Samples were consecutively sectioned at thickness of 10 μm in a cryostat unit at -15 to -18 °C. Sections were placed on statically charged slides and placed on a slide warmer for a minimum of 30 min and then stained with hematoxylin and counterstained with eosin. Morphologically distinct characteristics were identified for each sex and used to assign known males and females within the study (Luckenbach et al., 2003; Devlin and Nagahama, 2002). From these 50 samples, the female with the lowest $P450_{arom}$/ng cDNA value and the male with the highest $P450_{arom}/\mu$g cDNA were histologically verified based on the sex specific structures. Histology was then performed on 30 additional fish within the range of these 2 samples to identify a separation between males and females in $P450_{arom}$ expression.

Statistics

A two-way ANOVA was performed to examine the relationship between temperature, size group, temperature x group, and the proportion of females. Additionally, a linear regression was conducted to examine if there were significant differences in the proportion of females amongst treatments in the size range at which juveniles were exposed to treatment temperatures. A two-way nested ANOVA was conducted for each group on final TL to test if there were any significant differences in

the final size between temperature treatments and sexes. In addition, the two-way nested ANOVA determined if there was a tank effect on final TL. The data met all the assumptions of ANOVA and all statistical analyses were performed using SYSTAT v 10.0 (SPSS Inc., 2000 Chicago, IL).

RESULTS

Mean temperatures, salinity, and DO during the temperature exposure study were relatively constant within each treatment (Table 1). Mean DO was highest at 18 °C, and decreased across treatments with increases in temperature. In addition, mean salinity differed across treatments possibly due to differences in evaporation between temperatures. Copies of $P450_{arom}$/ ng cDNA across samples ranged from 0.0 to 862,998.7 (Figure 1). The lowest female expression verified by histology was 643.7 P450arom/cDNA, while the highest male expression verified by histology was 584.6 P450arom/cDNA (Figure 2). There is a narrow but definitive separation between the two sexes and therefore, all individuals with a P450arom/cDNA ≥ 643.7 were assigned female, and all individuals with a P450arom/cDNA ≤ 584.6 were assigned male. Distinctive characteristics in the gonads used to confirm each sex are shown in figures 3 and 4. The proportion of females across temperatures within each group is shown in Table 2. Histograms of the percentages of each sex per temperature for each size group were created (Figure 5).

In size group 1 (35-45 mm TL), the proportion of females decreased significantly as temperature increased; while in group 2 (65-70 mm TL), there is no significant pattern

of decrease in proportion of females with increased temperature. Temperature and the interaction between size group and temperature were significant on the proportion of females in the study (Table 3).

To further investigate the significance of size group and temperature on the proportion of females, separate linear regressions were run on each size group. There was a significant difference in proportion of females across temperatures depending on the size at exposure (Figure 6). In size group 1, there was a significant decrease in proportion of females as temperature increased (R^2= 0.917, p= 0.010), but in size group two, there was no clear trend, and the proportion of females across treatment temperatures were not significantly different from each other (R^2= 0.373, p= 0.269).

Mean final TL for each sex by group and temperatures are reported in Table 4. The two-way nested ANOVA for each group verified that there was no significant effect of tank on final TL in group 1 (p = 0.422) or group 2 (p = 0.986) (Table 5). In group 1, there were significant differences in final TL across temperatures (p = 0.000) but not between sexes (p = 0.816). In group 2, however, there were significant differences in final total length across temperatures (p = 0.001) and between sexes (p = 0.005).

DISCUSSION

This research identifies that sex is influenced by temperature between 35 and 65 mm TL and establishes that increases in temperature from 18 °C during this size range produce increasingly male skewed sex ratios in southern flounder from Texas. While temperature plays a large role in sex determination of southern flounder, it is unclear

what other environmental factors may have played a role. Mean DO levels decreased as temperature increased but were maintained very consistently throughout the study. Previous work on similar sized juvenile southern flounder has shown that growth was significantly reduced at DO concentration of 2.8 mg/L but not at 4.7 mg/L (Taylor and Miller, 2001). The lowest concentration measured in this study was 5.19 mg/L at 29.6 °C. Therefore, DO concentrations were not believed to have had an effect on the wellbeing or sex determination of juvenile southern flounder. It has recently been shown that other environmental factors such as lighting and tank color can elevate cortisol levels which is associated with producing an increased percentage of male southern flounder (Turner, 2008). In addition, southern flounder from North Carolina and Texas exhibited near 1:1 sex ratios at different temperatures in the laboratory, but were reared in freshwater and full strength seawater respectively. Thus, differences in salinity between these two studies may have an effect on sex determination, though more information is need.

Temperatures producing near 1:1 sex ratios in the laboratory (23 and 18 °C respectively) for both North Carolina and Texas flounder populations are not representative of naturally occurring temperature variability experienced by juvenile southern flounder in the bays during the critical period where temperature can influence sex determination. Difficulties in replicating natural temperature fluctuations in the laboratory present a challenge in definitively determining naturally occurring sex ratios of southern flounder. Though studies in this area are extremely limited, it has been

suggested that wild sex ratios for southern flounder are 1:1 in North Carolina (Luckenbach et al., 2005) and are female skewed in Texas (Stunz, et al., 2000).

A near 1:1 proportion of males and females is seen at 18 °C in Texas flounder in captivity, and it appears that warmer temperatures substantially induce male differentiation. Different influences of specific temperatures on phenotypic sex between these two populations indicates that there are latitudinal differences in sex determination in southern flounder perhaps as a result of adaptation to local environments. Latitudinal differences in sex determining mechanisms have been shown in other species including the Atlantic silverside, *Menidia menidia,* where more northerly latitudes exhibit GSD with little influence of temperature, and southerly latitudes exhibit a lesser degree of GSD and a higher degree of TSD (Conover and Kynard, 1981; Conover, 1984).

The significant differences in mean TL due to temperature for group 1 and temperature and sex for group 2 is consistent with the hypotheses of the adaptive significance of ESD proposed by Charnov and Bull, 1977, which suggests ESD supports differences in growth between sexes in optimal growth conditions to maximize reproductive success. This hypothesis has been supported in previous studies of both Atlantic silversides and southern flounder (Conover, 1984; Luckenbach et al., 2003). Interestingly, the optimal rearing temperature of 18°C for larval southern flounder from Texas (Faulk and Holt personal communication) also produced the highest proportion of females. This paradigm is consistent with the pattern for southern flounder from North Carolina (Luckenbach et al., 2003), where the optimum rearing temperature for larvae (23°C) also produces the greatest proportion of females. The differences identified in sex

ratios across temperatures may indicate that southern flounder exhibit site-specific adaptation, or that Atlantic and Gulf of Mexico stocks may be more genetically distinct than previously thought.

Water temperatures have increased in the last decade for South Texas (Texas Coastal Ocean Observation Network [TCOON], 2009). The Intergovernmental Panel for Climate Change (IPCC), Synthesis Report for 2007, predicts a 2-3 °C increase in climate for North America for the next 50 to 100 years. This may have a negative effect on already decreasing populations of southern flounder throughout their range as warmer temperatures produce male skewed sex ratios. An increase in temperature may also lead to a shift in the starting point and duration of the winter spawning period of southern founder. If the spawning period is delayed, juvenile southern flounder may enter the bays and estuaries later in the year. This exposure to warmer spring temperatures during sex determination could cause a shift in naturally occurring sex ratios and potentially reduce the number of new female recruits to future populations.

In terms of production, raising juvenile southern flounder from Texas at a steady temperature of 18°C throughout 65 mm TL in captivity produced a nearly equal proportion of males and females in this study. As a result, increasing growout temperatures to increase growth rates would need to be done after 65mm to maximize the number of females produced. The trend of fewer females with increased temperature seen in southern flounder from both North Carolina and Texas suggests that climate change may have negative effects on already threatened populations of southern flounder. With the sustainability of wild southern flounder populations already in question, this

study calls attention to the urgency of improved management strategies to protect and ensure southern flounder for the future.

Temperature °C	**Salinity (ppt)**	**D.O. (mg/L)**
18.2 ± 0.3	32.5 ± 1.5	7.8 ± 0.6
21.0 ± 0.5	33.6 ± 1.5	7.5 ± 0.6
23.8 ± 0.4	33.1 ± 1.5	6.9 ± 0.6
26.5 ± 0.5	34.0 ± 1.2	6.5 ± 0.6
29.6 ± 0.5	33.4 ± 1.3	5.7 ± 0.6

Table 1. Mean and standard deviation of rearing parameters during temperature treatments.

Temperature (°C)	TL (I) (mm)	TL (F) (mm)	Proportion Female
Group 1 (35-40 mm TL)			
18.2 ± 0.3	37.4 ± 1.7	101.6 ± 10.8	0.48 ± 0.11
21.0 ± 0.5	37.9 ± 1.5	98.8 ± 13.8	0.30 ± 0.21
23.8 ± 0.4	38.9 ± 1.3	100.6 ± 10.0	0.31 ± 0.10
26.5 ± 0.5	38.5 ± 1.6	115.2 ± 15.2	0.21 ± 0.07
29.6 ± 0.5	38.4 ± 1.8	104.3 ± 17.3	0.05 ± 0.00
Group 2 (65-70 mm TL)			
18.2 ± 0.3	62.6 ± 1.6	106.9 ± 12.3	0.43 ± 0.14
21.0 ± 0.5	68.0 ± 1.8	101.4 ± 8.6	0.33 ± 0.05
23.8 ± 0.4	68.2 ± 1.9	106.2 ± 10.3	0.36 ± 0.01
26.5 ± 0.5	67.4 ± 1.9	108.9 ± 10.0	0.31 ± 0.10
29.6 ± 0.5	66.0 ± 1.6	100.2 ± 8.7	0.33 ± 0.12

Table 2. Mean total length and standard deviation for temperature and size groups prior to temperature exposure (TL (I)) and at the end of the study (TL (F)), and proportion females with standard deviation as determined by qRT-PCR and cDNA quantification.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
TEMP	0.133	1	0.133	14.705	0.001
GROUP	0.038	1	0.038	4.253	0.056
GROUP*TEMP	0.054	1	0.054	5.945	0.027
Error	0.145	16	0.009		

Table 3. Two-way ANOVA of temperature (TEMP) and size group (GROUP) on proportion of females. $P < 0.05$ represents a significant effect on proportion of females.

Temperature (°C)	TL (F)	Sex
Group 1 (35-40 mm TL)		
18.2 ± 0.3	105.0 ± 11.20	F
18.2 ± 0.3	98.45 ± 9.70	M
21 ± 0.5	107.25 ± 14.10	F
21 ± 0.5	95.21 ± 12.20	M
23.8 ± 0.4	100.22 ± 10.70	F
23.8 ± 0.4	100.73 ± 9.80	M
26.5 ± 0.5	115.80 ± 18.40	F
26.5 ± 0.5	114.97 ± 14.60	M
29.6 ± 0.5	86.70 ± 7.50	F
29.6 ± 0.5	105.18 ± 17.20	M
Group 2 (65-70 mm TL)		
18.2 ± 0.3	112.08 ± 12.90	F
18.2 ± 0.3	102.72 ± 10.20	M
21 ± 0.5	104.05 ± 8.50	F
21 ± 0.5	100.05 ± 8.60	M
23.8 ± 0.4	111.41 ± 10.70	F
23.8 ± 0.4	103.22 ± 9.00	M
26.5 ± 0.5	108.49 ± 13.70	F
26.5 ± 0.5	109.06 ± 7.90	M
29.6 ± 0.5	100.63 ± 10.80	F
29.6 ± 0.5	100.06 ± 7.60	M

Table 4. Mean final total length (TL (F)) and standard deviation for each sex by group and temperature.

Source	Sum-of-squares	df	Mean-square	F-ratio	P
Group 1 (35-40 mm TL)					
SEX	9.744	1	9.744	0.054	0.816
TEMP	4,728.18	4	1182.046	6.573	0.000
TEMP*SEX	1,370.17	4	342.541	1.905	0.112
TANK (TEMP)	894.523	5	178.905	0.995	0.422
Error	32,730.33	182	179.837		
Group 2 (65-70 mm TL)					
SEX	775.559	1	775.559	7.923	0.005
TEMP	1,936.74	4	484.184	4.946	0.001
TEMP*SEX	664.274	4	166.068	1.696	0.153
TANK (TEMP)	62.926	5	12.585	0.129	0.986
Error	17,718.63	181	97.893		

Table 5. Two-way nested ANOVA of sex (SEX), temperature (TEMP), and replicate tanks nested within temperature (TANK (TEMP)). $P < 0.05$ represents a significant effect on final total length.

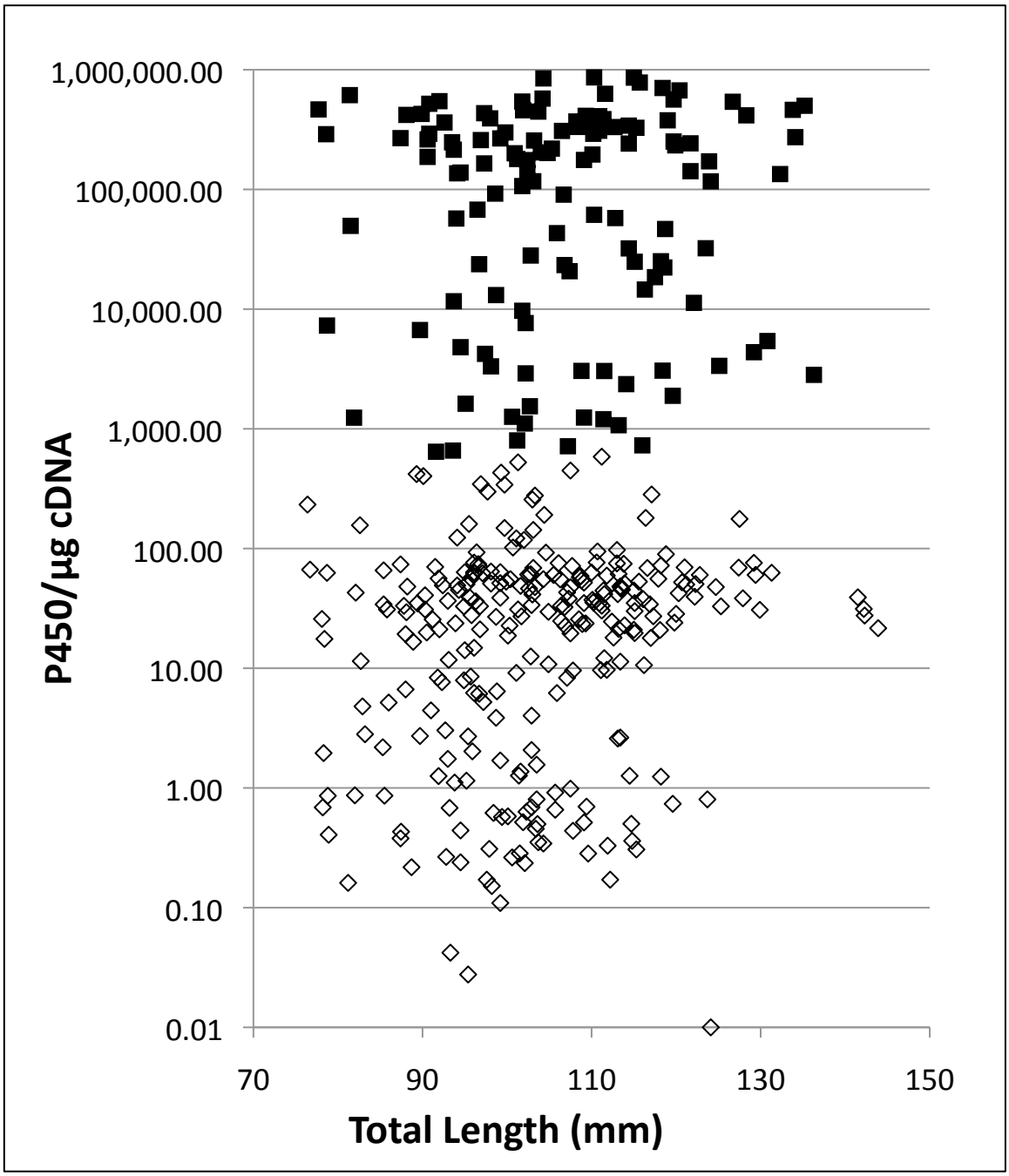


Figure 1. Putative males (open diamonds) and females (black squares) plotted by final total length and copies of P450 per μg cDNA. The Y-axis has been modified to a logarithmic scale to include all samples.

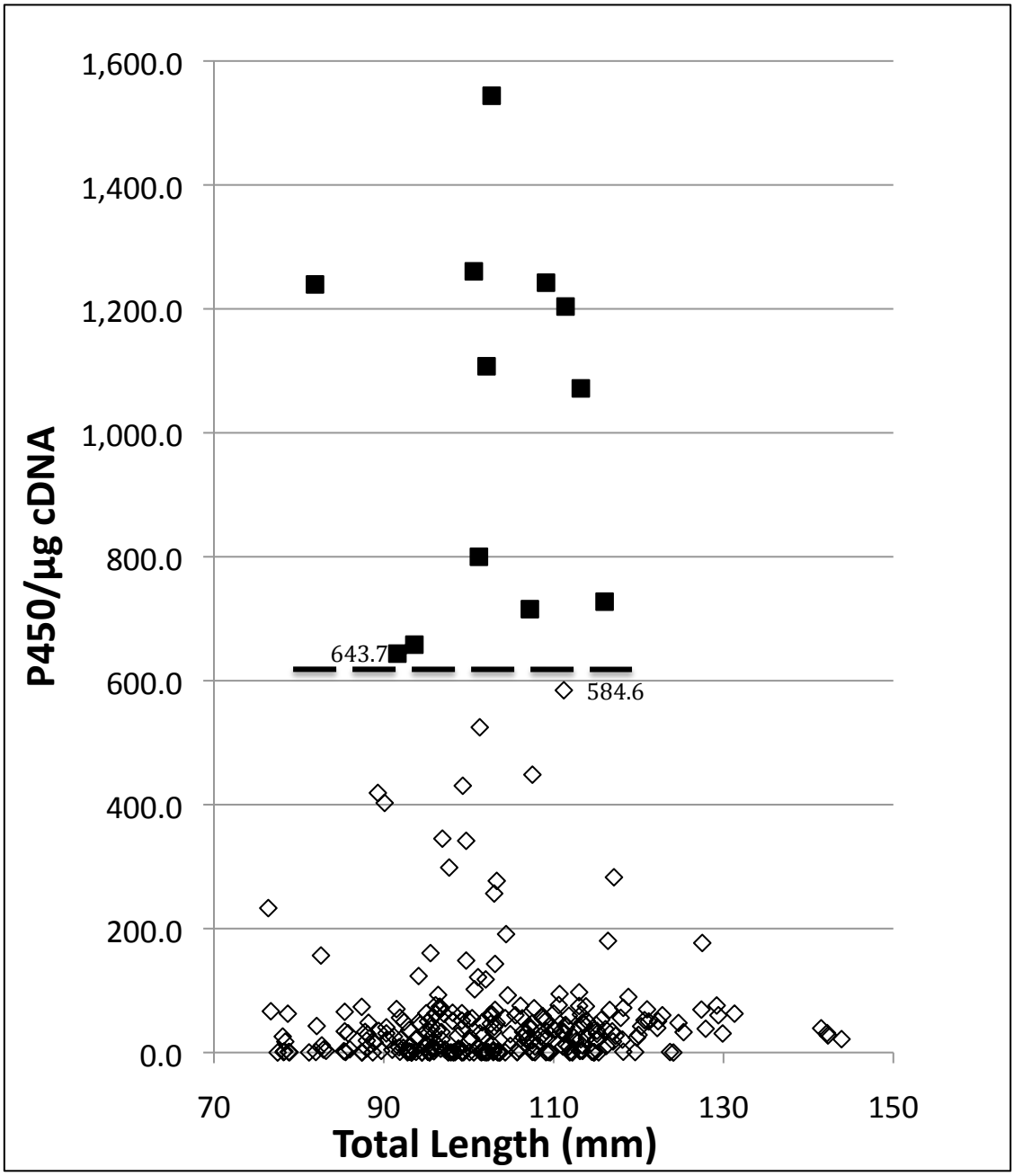


Figure 2. Putative males (open diamonds) and females (black squares) plotted by final total length and copies of P450 per μg cDNA. The Y-axis has been reduced to clearly show the separation between putative males and females by P450/μg cDNA expression. The lowest measured level in a female was 643.7 and the highest level measured in a male was 584.6. The dashed line illustrates the separation between sexes.

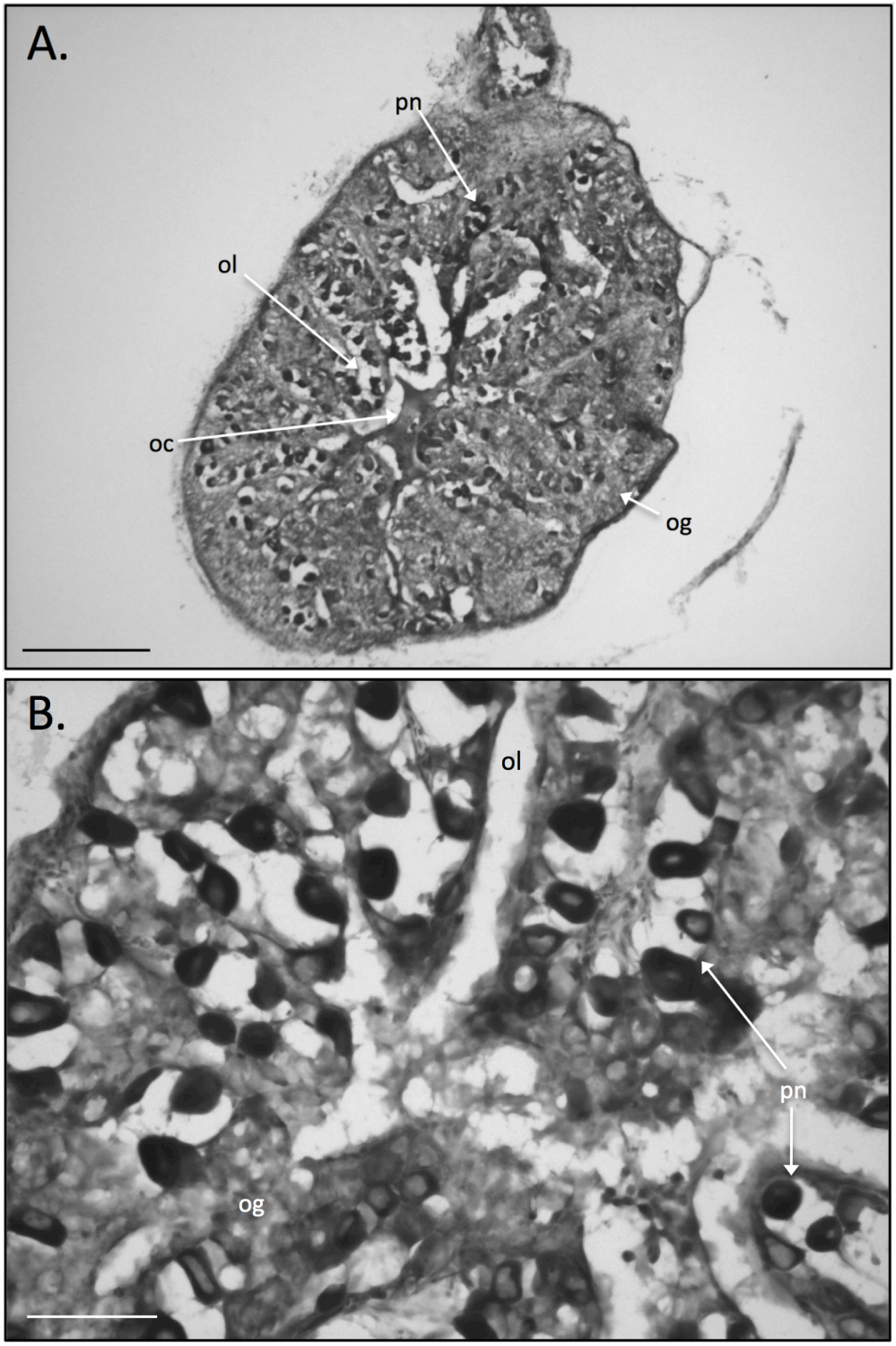


Figure 3. Ovarian differentiation in juvenile southern flounder. (A) Cross section of a gonad from a 127.6 mm TL fish magnified at 100X. oc, ovarian cavity, ol, ovarian lumen, og, oogonium, pn, perinucleolus stage oocytes. The black bar = 20 μm. (B) Cross section of a gonad from a 98 mm TL fish. The same structures are shown at a higher magnification. The white bar = 5 μm.

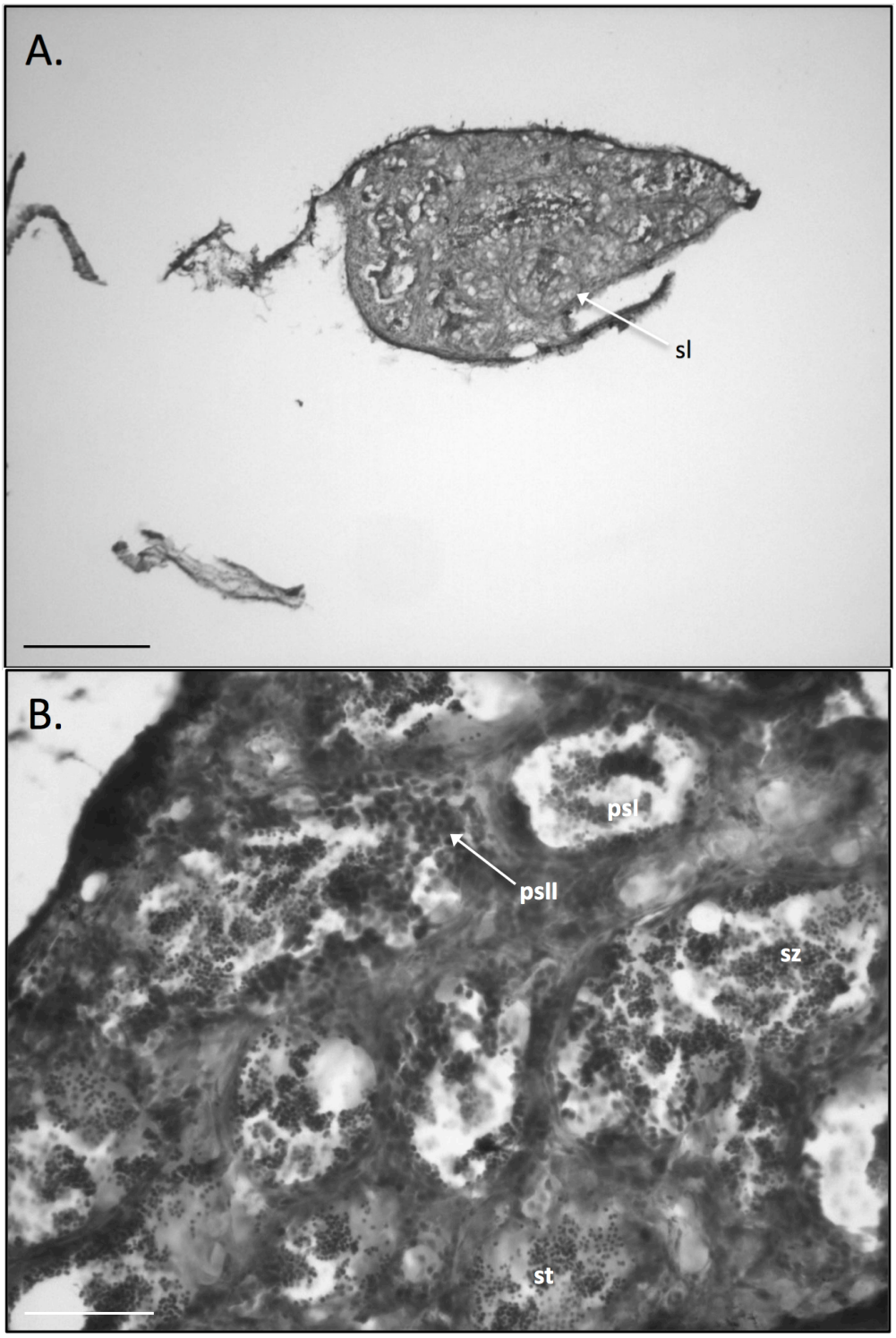
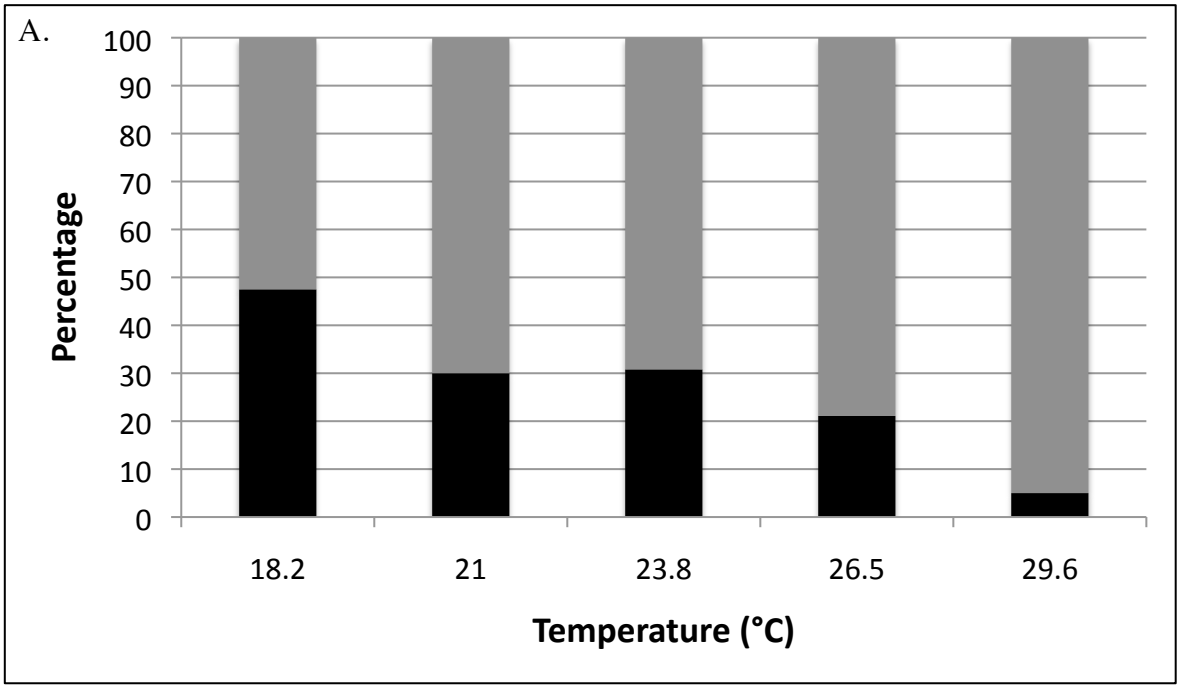


Figure 4. Testicular differentiation in juvenile southern flounder. (A) Sagittal section of a gonad from a 103.1 mm TL fish. sl, seminal lobule with sg, spermatigonium at various stages. The black bar = 20 μm. (B) Cross section of a gonad from a 111.2 mm TL fish. sl are shown with clusters of st, spermatids, sz, spermatazoa, psI, primary spermatocytes, psII, secondary spermatocytes. The white bar = 5 μm.



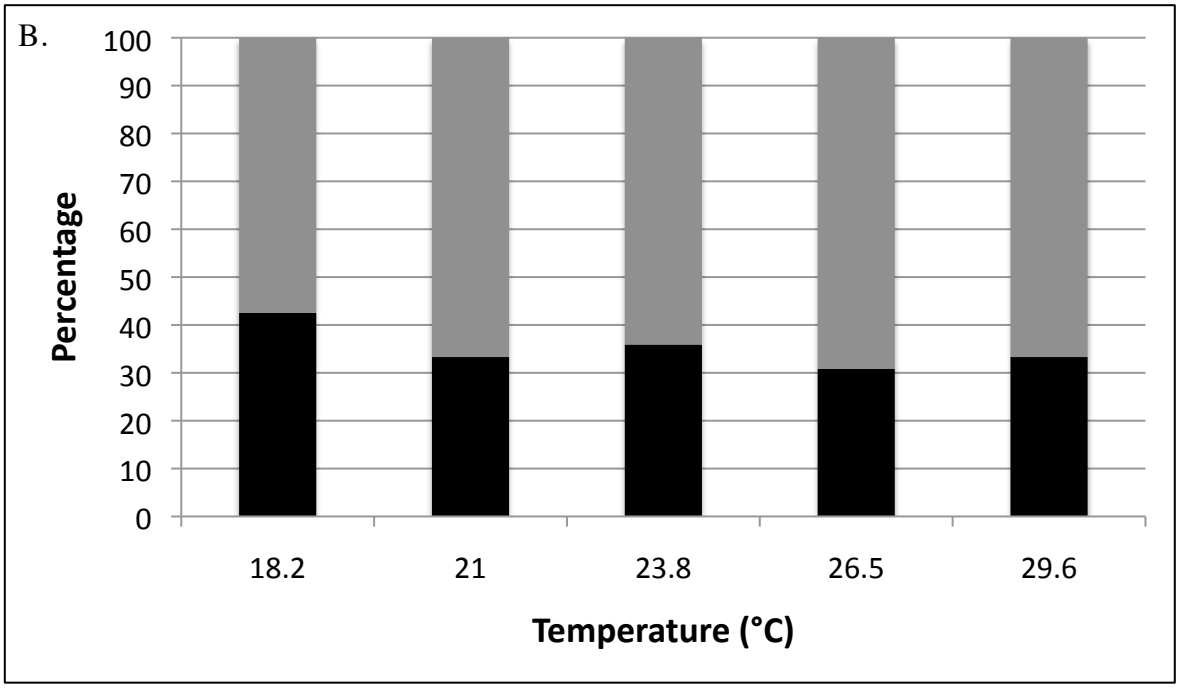


Figure 5. Effects of temperature and size on sex determination in juvenile southern flounder from Texas. Mean percentages of males (grey) and females (black) produced from replicates of each size group (A) 35-40mm TL and (B) 65-70 mm TL at temperatures of 18.2, 21.0, 23.8, 26.5, and 29.6 °C.

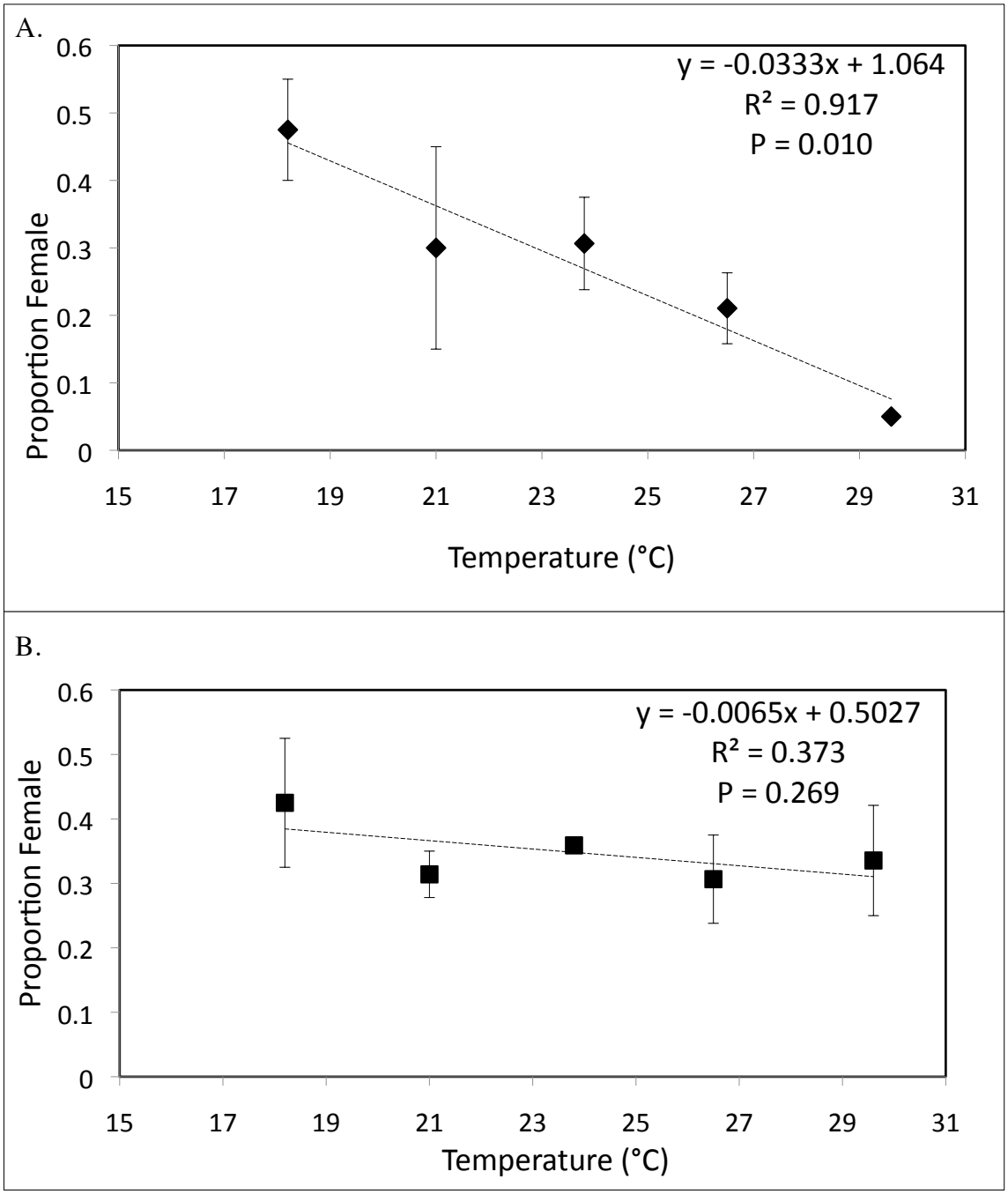


Figure 6. Linear regression of the effects of temperature and size on sex determination in juvenile southern flounder from Texas. The proportion of females produced by size group (A) 35-40 mm TL and (B) 65-70 mm TL at temperatures of 18.2°, 21.0°, 23.8°, 26.5°, and 29.6 °C.

References

Arnold, C.R., Bailey, W.H., Williams, T.D., Johnson, A., Lasswell, J.L., (1977). Laboratory spawning and larval rearing of red drum and southern flounder. Proc. Annu. Conf. Southeast. Assoc. Fish Wildl. Agencies 31, 437–440.

Blandon, I.R., Ward, R., King, T.L., Karel, W.J., and Monaghan, J.P. (2001). Preliminary genetic population structure of southern flounder, *Paralichthys lethostigma,* along the Atlantic Coast and Gulf of Mexico. *Fisheries Bulletin*, 99, 671-679.

Charnov E.L. and Bull, J.J. (1977). When sex is environmentally determined. *Nature,* 266, 828-830.

Conover, D.O. and Kynard, B.E. (1981). Environmental sex determination: Interaction of temperature and genotype in a fish. *Science*, 213(4507), 577-579.

Conover, D.O. (1984). Adaptive significance of temperature-dependent sex determination in a fish. *The American Naturalist*, 123(3), 297-313.

Conover, D.O. (2004). Temperature-dependent sex determination in fishes. In: Valenzuela, N., & Lance, V., (Eds.). *Temperature-dependent sex determination in Vertebrates* (11-20). Washington: Smithsonian Institution Press.

Crews, D. (2003). Sex determination: Where environment and genetics meet. *Evolution and Development,* 5(1), 50-55.

Daniels, H.V. (2000). Species profile southern flounder. *Southern Regional Aquaculture Center,* 726.

Devlin, R.H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture,* 208, 191-364

Faulk, C.K., Kaiser, J.B., and Holt, G.J. (2007). Growth and survival of larval and juvenile cobia *Rachycentron canadum* in a recirculating raceway system. *Aquaculture,* 270(1-4), 149-157.

Faulk, C.K. and Holt, G.J. (2009). Early weaning of southern flounder, *Paralichthys lethostigma,* larvae and ontogeny of selected digestive enzymes. *Aquaculture,* 296, 213-218.

Faulk, C.K., Pérez-Domínguez, R., Webb Jr., K.A., and Holt, G.J. (2010). The novel finding of four distince prepro-IGF-I E domains in a perciform fish, *Sciaenops ocellatus*, during ontogeny. *General and Comparative Endocrinology*, 169, 75-81.

Frederieke, J.K., Munday, P.L., Westcott, D.A., Hobbs, J.A., and Liley, N.R. (2005). Aromatase pathway mediates sex change in each direction. *Proceedings of the Royal Society,* 272, 1399-1405.

Glass, L.A., Rooker, J.R., Kraus, R.T., and Holt, G.J. (2008). Distribution, condition, and growth of newly settled southern flounder (*Paralichthys lethostigma*) in the Galveston Bay Estuary, TX. *Journal of Sea Research,* 59(4), 259-268.

GSMFC. (2000). The flounder fishery of the Gulf of Mexico, United States: A regional management plan. Publication No. 83. Gulf States Marine Fisheries Commission. Ocean Springs, Mississippi.

Holt, G.J. (2002). Human impacts. In: Fuiman, L.A., & Werner, R.G. (Eds). *Fishery Science: The Unique Contributions of Early Life Stages* (222-242). Oxford: Blackwell Science Ltd.

Intergovernmental Panel on Climate Change (IPCC). (2007). *Climate change 2007: Synthesis report, Contribution of working groups I, II, and III to the fourth assessment report of the Intergovernmental Panel on Climate Change,* [Core Writing Team, R. K. Pachauri & A Reisinger, (Eds.)]. IPCC< Geneva, Switzerland.

Kleypas, J. and Hoegh-Guldberg, O. (2008). Coral reefs and climate change: Susceptibility and consequences. In: Wilkinson, C., & Souter, D. (Eds.). *Status of Caribbean Coral Reefs After Bleaching and Hurricanes in 2005* (19-28). Townsville: Global Coral Reef Monitoring Network, and Reef and Rainforest Research Centre.

Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., and Abe, S.I. (1999). Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). *Journal of Molecular Endocrinology,* 23(2), 1-10.

Lagomarsino, I.V. and Conover, D.O. (1993). Variation in environmental and genotypic sex-determining mechanisms across a latitudinal gradient in the fish, *Menidia menidia*. *Evolution*, 47(2), 487-494.

Libus, J. and Štorchová, H. (2006). Quantification of cDNA generated by reverse transcription of total RNA provides a simple alternative tool for quantitative RT-PCR normalization. *BioTechniques*, 41, 156-164.

Luckenbach, J.A., Godwin, J., Daniels, H.V., and Borski, R.J. (2003). Gonadal differentiation and effects of temperature on sex determination in southern flounder (*Paralichthys lethostigma*). *Aquaculture,* 216, 315-327.

Luckenbach, J.A., Godwin, J., Daniels, H.V., Beasley, J.M., Sullivan, C.V., Borski, R.J. (2004). Induction of diploid gynogenesis in southern flounder (*Paralichthys lethostigma*) with homologous and heterologous sperm. *Aquaculture*, 237, 499-515.

Luckenbach, J.A., Early, L.W., Rowe, A.H., Borski, R.J., Daniels, H.V., and Godwin, J. (2005). Aromatase cytochrome P450: Cloning, intron variation, and ontogeny of gene expression in southern flounder (*Paralichthys lethostigma*). *Journal of Experimental Zoology,* 303A, 643-656.

Nañez-James, S.E., Stunz, G.W., and Holt, S.A. (2009). Habitat use patterns of newly settled southern flounder, *Paralichthys lethostigma*, in Aransas-Copano Bay, Texas. *Estuaries and Coasts*, 32, 350-359.

O'Connor, M.I., Bruno, J.F., Gaines, S.D., Halpern, B.S., Lester, S.E., Kinlan, B.P., et al. (2007). Temperature control of larval dispersal and the implications for marine ecology, evolution, and conservation. *Proceedings of the National Academy of Sciences,* 104(4), 1266-1271.

Sala, O.E., et al. (2000). Global biodiversity scenarios for the year 2100. *Science,* 287, 1770-1774.

Stokes, G.M. (1977). Life history studies of southern flounder (*Paralichthys lethostigma*) and Gulf flounder (*P. albigutta*) in the Aransas Bay area of Texas. *PWD Bulletin,* 3000-3033.

Takade-Heumacher, H. and Batsavage, C. (2009). Stock status of North Carolina southern flounder (*Paralichthys lethostigma*). *North Carolina Division of Marine Fisheries*, Morehead City, NC.

Taylor, J.C., and Miller, J.M. (2001). Physiological performance of juvenile southern flounder, *Paralichthys lethostigma* (Jordan and Gilbert, 1884), in chronic and episodic hypoxia. *Journal of Experimental Marine Biology and Ecology,* 258, 195-214.

Texas Coastal Ocean Observation Network. (2009). Basic Query Parameters for Aransas Copano Bay. Retrieved from http://lighthouse.tamucc.edu/pq/009.

Turner, P.E. (2008). Effects of light intensity and tank background color on sex determination in souther flounder (*Paralichthys lethostigma*). Retrieved from NCSU Digital Repository http://www.lib.ncsu.edu/resolver/1840.16/1482.

Walther, G.R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T.J.C., et al. (2002). Ecological responses to recent climate change. *Nature*, 416, 389-395.

Wenner, C. and Archambault, J. (2005). Southern flounder: Natural history and fishing techniques in South Carolina. *South Carolina Department of Natural Resources,* Charleston, SC.

Vita

Avier José Montalvo was born in Port Lavaca, Texas, USA on December 5, 1982, the son of Edris Montalvo Sr. and Lillian Montalvo. After graduating from Calhoun High School in Port Lavaca, Texas in 2001, he entered Texas State University, San Marcos, Texas. After the marine biology program was discontinued, he transferred to Texas A&M University at Galveston, Galveston, Texas where he obtained a Bachelor of Science in Marine Fisheries in December of 2007. In August 2008, he entered the Master's degree program in the Department of Marine Science at the University of Texas at Austin.

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